Enhancement of Cisplatin Sensitivity in Squamous Cell Carcinoma of the Head and Neck Transfected With a Survivin Antisense Gene

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Objective: To study a new method for treating squamous cell carcinoma of the head and neck using a survivin antisense gene.

Design: An adenoviral vector encoding survivin antisense was used for in vitro and in vivo experiments. KB cells were treated with pAd.CMV[cytomegalovirus]-antisurvivin. Western blot analysis, in vitro cytotoxic assay, and in vivo experiment were performed.

Setting: In vitro and in vivo study of head and neck cancer cell line KB.

Subjects: Male, 5-week-old BALB/c nude mice.

Main Outcome Measures: Expression of survivin was assessed using Western blot analysis. The effect of antisurvivin to KB cells was measured by cytotoxic assay (in vitro) and tumor volume (in vivo).

Results: In the in vitro experiments, transduction of the survivin antisense gene caused a nearly 12-fold increase in the sensitivity of KB cells to cisplatin, as reflected by the 50% inhibitory concentration. In in vivo experiments in nude mice, tumor growth was more inhibited by the combination of cisplatin and survivin antisense gene transduction compared with either alone.

Conclusion: These findings suggest that survivin targeting with adenoviral antisense vectors might be used for selective therapy of squamous cell carcinomas of the head and neck.

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Local control of head and neck squamous cell carcinoma (HNSCC) is essential, and administering anticancer drugs directly into various lesion sites by injection is comparatively easy. Therefore, genetic therapy is considered to have an enormous potential benefit. Now being investigated for the treatment of HNSCC are multiple gene transfer strategies that involve the introduction of genes that directly kill tumor cells, restore a defective tumor-suppressor gene, or induce apoptosis; genes that enhance the immune response; and anticancer drug-sensitivity genes that encode an enzyme that can activate a prodrug within tumors.1,2

Recently, a family of antiapoptotic cellular genes related to the baculovirus iap gene has been described.3,4 Members of the inhibitor of apoptosis protein (IAP) family inhibit apoptosis in different contexts,3 although their mechanism of action is not completely understood. A novel member of the IAP gene family, designated survivin,6 was recently identified by hybridization screening of human genomic libraries with the complementary DNA (cDNA) of a factor Xa receptor, effector cell protease receptor 1.7 Unlike all other IAPs, survivin is expressed during development and by common human cancers but is undetectable or detected at extremely low levels in normal adult tissues.6 Interestingly, survivin is reexpressed in transformed cell lines and a variety of human tumors.8 Survivin inhibits apoptosis induced by interleukin3 withdrawal in B-cell precursors. In several tumor series, the presence of survivin was correlated with a lower apoptotic index in vivo, shorter overall survival, unfavorable prognosis, and higher rates of recurrences.9,10 The high prevalence of survivin in many human cancers has prompted studies using survivin as a therapeutic target in the treatment of cancer and as a prognostic marker for cancer. In addition, survivin deserves attention as a selective target for cancer therapy because it is not expressed in differentiated adult tissues but is expressed in a variety of human tumors.

Recently, molecular antagonists of survivin, including antisense genes and expression of dominant negative mutants, have been used to induce spontaneous apoptosis of cancer cells in vitro11 and in vivo12 and to enhance chemotherapy-induced cell death.13 In the present study, we investigated the effects of a survivin antisense gene on HNSCC. We designed a natural antisense gene to survivin and investigated its ability to down-regulate...
units (PFU) assayed in semisolid cultures of HEK293 cells.

stayed at −80°C. Viral titers were determined as plaque-forming units on monolayers of HEK293 cells grown in 100-mm diameter dishes treated with medium containing 10% fetal bovine serum, penicillin at 100 U/mL, streptomycin sulfate at 100 µg/mL, and 2mM of L-glutamine.

The experimental animals used were male, 5-week-old BALB/c nude mice (Clea Japan Inc, Tokyo, Japan).

CONSTRUCTION OF ADENOVIRAL VECTORS

The replication-defective adenoviral vectors encoding an antisense gene to survivin RNA was constructed by one of us (Y.M.) and Tetsuhsia Yamamoto, MD, Department of General and Gastroenterological Surgery, Osaka Medical College, Osaka, Japan. The vector was constructed by homologous recombination of a 788 base pair sequence of the human survivin RNA containing the antisense RNA sequence complementary to survivin mRNA.

TUMOR CELL LINES AND ANIMALS

Human KB floor-of-mouth squamous cell carcinoma cells (Japanese Collection of Research Bioresources, Osaka, Japan) were grown as a monolayer in Dulbecco modified Eagle minimum essential medium supplemented with 10% inactivated fetal bovine serum, penicillin at 100 U/mL, streptomycin sulfate at 100 µg/mL, and 2mM of L-glutamine.

Survivin expression in KB cells transduced with pAd.CMV-antisurvivin (2 × 108 PFU/50 µL) or viral vehicle (50 µL).

KB cells (1 × 106 cells/50 µL) were injected subcutaneously to the backs of nude mice, and engraftment was confirmed when the long tumor diameter reached approximately 4 mm on the fifth day after injection. Then, pAd.CMV-antisurvivin (2 × 108 pfu/50 µL) was injected intratumorally. The control groups received intratumoral injections of pAd.CMV-LacZ (2 × 108 PFU/50 µL) or viral vehicle (50 µL).

Forty-eight hours after viral transfection, cisplatin (2 mg/kg) or phosphate-buffered saline was injected into the peritoneal cavity of the animals, and the volume of the tumor was determined with the passage of time. The tumor volume was calculated as the product of its long and short diameters. All animal procedures were performed under the guidance of the committee of our animal care facility.

IN VITRO CYTOTOXIC ASSAY

The adenovirus vector pAd.CMV-antisurvivin, to which the survivin antisense gene had been transduced, was transfected into KB cells at a multiple of infectivity (MOI) of 2 or 10. KB cells treated with viral vehicle or infected with 10 MOI of pAd.CMV-LacZ were used as controls. The viral vehicle consisted of the lysate of the adenovirus. After 48 hours, the transfected cells were fixed after cisplatin exposure and stained with 0.05% methylene blue. The dye was eluted with 0.33 N hydrochloric acid for 15 minutes with agitation. Absorbance was measured with a microtiter reader (model 550; Bio-Rad Laboratories, Hercules, Calif) at 595 nm. Values were determined within the linear range and standardized to a control curve.

RESULTS

ADENOVIRUS-MEDIATED TRANSDUCTION OF KB CELLS WITH THE ANTISURVIVIN GENE

We first examined survivin expression in various kinds of squamous cell carcinoma cell lines. Survivin was expressed in all cell lines (Figure 1). Next, KB cells were transduced with pAd.CMV-antisurvivin (MOI = 2 or 10) for 48 hours and harvested, and the expression of the survivin protein was demonstrated with Western blot analysis. Survivin was expressed in untreated KB cells, but the expression was decreased in KB cells transduced with pAd.CMV-antisurvivin.

To determine whether pAd.CMV-antisurvivin has biological activity in vitro, we infected KB cells with 2 or 10...
These values indicate that the sensitivity of KB cells to antisurvivin at an MOI of 2 and 2 µM at an MOI of 10. LacZ (MOI=10) was 25 µM but was 9 µM with pAd.CMV-infected with pAd.CMV-antisurvivin. cisplatin caused significant killing of cells that had been transduced with the survivin antisense gene inhibited tumor growth. Bars show the standard deviations.

Figure 3. Cytotoxic assay showing the sensitivity of KB cells to cisplatin after adenoviral vector transduction. KB cells were infected at various multiple of infectivities (MOIs) with the recombinant adenoviruses pAd.CMV-antisurvivin (MOI=2 or 10) and pAd.CMV-LacZ (MOI=10). After 48 hours, cells were exposed to the indicated concentration of cisplatin for an additional 48 hours. The results are expressed as the mean of 8 experiments; bars show the standard deviations. Cell killing was significantly greater with pAd.CMV-antisurvivin at an MOI of 10 (P<.01).

Figure 4. Effects of pAd.CMV[cytomegalovirus]-anti-survivin against KB tumors in vivo. KB cells (1 × 10⁶) were injected on day 0. Five days later, viral vehicle or 2 × 10⁸ plaque-forming units (PFU) of pAd.CMV-antisurvivin or pAd.CMV-LacZ was injected into the tumors. Two days later, mice were treated with either phosphate-buffered saline or cisplatin. Transduction of the survivin antisense gene inhibited tumor growth. Bars show the standard deviations.

MOI of virus. Forty-eight hours after viral transduction, the cells were plated in 96-well plates at a density of 2 × 10³ cells per well and exposed to various concentrations of cisplatin for 48 hours. Whereas cisplatin caused only modest cell death of KB cells treated with viral vehicle or infected with pAd.CMV-LacZ control virus (MOI=10), cisplatin caused significant killing of cells that had been infected with pAd.CMV-antisurvivin (P<.01) (Figure 3).

The 50% inhibitory concentration (IC₅₀) with pAd.CMV-LacZ (MOI=10) was 25 µM but was 9 µM with pAd.CMV-antisurvivin at an MOI of 2 and 2 µM at an MOI of 10. These values indicate that the sensitivity of KB cells to cisplatin was increased approximately 12-fold by the transduction of the survivin antisense gene at an MOI of 10.

Figure 5. Effects of the combination of pAd.CMV[cytomegalovirus]-antisurvivin and cisplatin in an in vivo experiment. The combination of antisurvivin gene transduction and cisplatin administration significantly inhibited tumor growth. PBS indicates phosphate-buffered saline; PFU, plaque-forming unit. Bars show the standard deviations.

TREATMENT OF KB CELL-TUMOR WITH pAd.CMV-ANTISURVIVIN

Nude mice with tumors received injections of pAd.CMV-antisurvivin, pAd.CMV-LacZ, or viral vehicle and then were treated with phosphate-buffered saline or cisplatin. The sizes of tumors were then measured.

Intratumor administration of pAd.CMV-LacZ had no effect on exponential tumor growth for up to 15 days after injection. In contrast, a single intratumor injection of pAd.CMV-antisurvivin inhibited tumor growth by approximately 60% on day 15 after injection (P<.01; Figure 4).

In addition, the tumor growth in mice treated with pAd.CMV-antisurvivin and cisplatin was inhibited to a much greater extent than that in mice treated with pAd.CMV-LacZ and cisplatin (Figure 4). All differences were statistically significant (Figure 5).

COMMENT

Diminished apoptosis plays an important role in tumor initiation, progression, and drug resistance. The major apoptosis-signaling pathways are the mitochondrial pathway and the death receptor pathway. Several proteins that inhibit apoptosis have been identified, including the members of the bcl-2 family, such as bcl-2 and bcl-xL, and the IAPs. The antiapoptotic proteins bcl-2 and bcl-xL block the apoptotic event of mitochondrial cytochrome c release into the cytosol and have been shown to inhibit mainly these 2 pathways.

The gene encoding the IAP survivin was recently cloned, and the protein was characterized. Survivin is thought to be expressed in the G2/M phase of the cell cycle in a cell cycle–regulated manner and to be associated with microtubule formation of the mitotic spindle.
Because survivin inhibits processing of the downstream effectors caspase-3 and caspase-7, which act at a common downstream part of the 2 major apoptosis pathways, its overexpression in tumors has been implicated in the resistance to a variety of apoptotic stimuli, including chemotherapy. For this reason, the survivin antisense gene might facilitate apoptosis via both pathways.

Although survivin has long been considered a potential target for cancer therapy, the use of antisense cDNA and oligonucleotides to inhibit its expression has only recently been described. Previous studies have shown that the reduction of survivin expression achieved by antisense strategies causes apoptotic cell death and sensitization to anticancer drugs in several tumor cell lines. These results suggest that survivin expression is likely important for cell survival or resistance to chemotherapy in carcinomas.

In this study, we have shown that a replication-deficient adenovirus encoding a survivin antisense gene down-regulates survivin expression and activity, causes spontaneous apoptosis in KB cells, and inhibits tumor growth in a model of HNSCC. These observations are consistent with an earlier finding that interaction between survivin and microtubules of the mitotic spindle apparatus is necessary to prevent a default induction of apoptosis at the G2/M phase of the cell cycle. Moreover, in a combination therapy experiment with cisplatin, we obtained evidence that antisense-mediated downregulation of survivin can sensitize tumor cells (KB cells) to chemotherapy in vitro and in vivo.

Cisplatin acts in the G2/M phase of the cell cycle. Previous studies have shown that an increase in chemosensitivity is negatively correlated with survivin expression and positively correlated with rates of apoptosis. Our results are consistent with the expression of survivin in the G2/M phase.

Our data suggest that the use of survivin antisense deserves further investigation as a useful approach to HNSCC cancer therapy.

In conclusion, a replication-deficient adenovirus encoding a survivin-antisense gene was shown to down-regulate survivin expression and activity, to cause apoptosis in KB cells, and to inhibit tumor growth. In addition, transduction of the survivin antisense gene enhances sensitivity to cisplatin. These findings indicate the potential of this combination of survivin antisense gene transduction and cisplatin administration as a new therapeutic strategy for HNSCC.

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REFERENCES


